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Effects of Immunosuppression with Cyclophosphamide on Acute Murine Cytomegalovirus Infection and Virus-Augmented Natural Killer Cell Activity

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16. ABSTRACT

The effects of cyclophosphamide (CY) treatment on acute murine cytomegalovirus (MCMV) infection were studied to explore the potential usefulness of MCMV as a means of detecting immune dysfunction and to identify host defense mechanisms imporant for protection against MCMV. Conditiops found optimal for enhancing MCMV infection with CY included infecting adult mice with $2x10^5$ PFU or more of virus and administering 80 mg or more of CY per kg 1 to 3 days later. In addition to enhanced mortality, virus titers in lung, liver, and spleen were elevated in CY in CY-treated mice, and wet weights of liver, spleen, and thymus were depressed when compared with those of infected but untreated mice. Treatment with CY before MCMV challenge was not as effcient a means of enhancing mortality as treatment after virus challenge. The effect that the time of CY administration relative to infection had on mortality correlated with the effect of such timing on natural killer cell activity. Animals treated before infection exhibited depressed natural killer cell activity initially. However, they rapidly recovered this response, and by 5 days postinfection they had the same level of virus-augmented activity seen in untreated mice. In contrast, animals treated after infection did not recover natural killer cell activity and were more likely to die. A similar correlation was not obtained when the effects of CY on lymphocyte responses to B and T cell mitogens were examined, nor were there striking differences in pathology between the treatment groups. The data suggest an imporant role for natural killer cells in host defense against MCMV.

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Effects of Immunosuppression with Cyclophosphamide on Acute Murine Cytomegalovirus Infection and Virus-Augmented Natural Killer Cell Activity

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The effects of cyclophosphamide (CY) treatment on acute murine cytomegalovirus (MCMV) infection were studied to explore the potential usefulness of MCMV as a means of detecting immune dysfunction and to identify host defense mechanisms important for protection against MCMV. Conditions found optimal for enhancing MCMV infection with CY included infecting adult mice with 2×10^5 PFU or more of virus and administering 80 mg or more of CY per kg 1 to 3 days later. In addition to enhanced mortality, virus titers in lung, liver, and spleen were elevated in CY-treated mice, and wet weights of liver, spleen, and thymus were depressed when compared with those of infected but untreated mice. Treatment with CY before MCMV challenge was not as efficient a means of enhancing mortality as treatment after virus challenge. The effect that the time of CY administration relative to infection had on mortality correlated with the effect of such timing on natural killer cell activity. Animals treated before infection exhibited depressed natural killer cell activity initially. However, they rapidly recovered this response, and by 5 days postinfection they had the same level of virus-augmented activity seen in untreated mice. In contrast, animals treated after infection did not recover natural killer cell activity and were more likely to die. A similar correlation was not obtained when the effects of CY on lymphocyte responses to B and T cell mitogens were examined, nor were there striking differences in pathology between the treatment groups. The data suggest an important role for natural killer cells in host defense against MCMV. Also, increased susceptibility to MCMV may provide a useful indicator of deficits in the natural killer cell response.

Although murine and human cytomegaloviruses usually produce benign infections in mature hosts, both are exacerbated by immunosuppressive treatment (5, 7, 9, 13, 14, 18, 20). Recent studies on the effects of immunosuppression on murine cytomegalovirus (MCMV) have focused on chronic or latent infections (7, 13, 14, 18, 19). However, several investigators have considered the effects of immunosuppressive treatment on the acute stage of infection (1, 10, 24), and the work presented here was undertaken to augment these studies. Our objectives were to explore the potential usefulness of MCMV infection as a means of detecting deficits in the immune response and to use immunosuppressive treatment to identify host defense mechanisms which are active in protecting against MCMV-induced disease.

A variety of tests are available to assess the functional integrity of various segments of the immune system. Such tests are commonly applied whenever it is suspected that host defense mechanisms are compromised by disease, exposure to drugs or other toxic chemicals, genetic defects, or other circumstances (2, 12, 22, 36). Due to the complexity of the immune response, no single assay is available to test the competence of the entire system, and it has been suggested that a tier of tests be developed, particularly when chemicals are to be screened for immunotoxicity (4, 36). Two of the most commonly used tests for immunocompetence are lymphocyte transformation by polyclonal mitogens and antibody responses to sheep erythrocytes. It is somewhat difficult to predict what the results of these tests mean in terms of enhanced risk of acquiring infectious, neoplastic, or autoimmune diseases. Susceptibility to

MCMV might provide a useful test which would directly measure enhanced susceptibility to an infectious agent and therefore be of value in a series of tests designed to measure immunotoxicity. To explore this possibility, we studied the effects of cyclophosphamide (CY), a known immunosuppressant, on mortality and pathogenesis during acute MCMV infection.

In addition, we studied the effects of CY on parameters used to assess the activity of certain segments of the immune defense system. We correlated the effects of CY on particular immune functions with effects on MCMV disease in an effort to elucidate the mechanisms which control acute MCMV and to determine what immunological functions must be depressed to enhance MCMV infection. Attention was focused on the functional integrity of lymphocytes with respect to blastogenic response and natural killer (NK) cell activity, since these are thought to be important in host defenses against cytomegaloviruses (3, 8, 15, 26, 27).

Although human cytomegalovirus infections after immunosuppression are largely attributable to reactivation of latent viruses, most studies indicate that infections also occur in previously seronegative individuals, who presumably experienced a primary infection while receiving immunosuppressive treatment (5, 6, 20, 21, 25). The present work with acute MCMV may provide insights into that particular clinical situation and into the host defense mechanisms which protect ordinary individuals from symptomatic human cytomegalovirus infections.

MATERIALS AND METHODS

Virus. The Smith strain of MCMV was obtained from the American Type Culture Collection (Rockville, Md.) and was passaged through mouse salivary glands. Virulent virus pools, which were used throughout this study, were obtained by making a 10% (wt/vol) extract of salivary glands 2 to 3 weeks postinfection. Detailed procedures for producing virus pools have been described previously (33). To determine infectivity titers of virus pools, plaque assays were carried out as follows. Confluent monolayers of secondary mouse embryo fibroblasts were produced in 25-cm^2 tissue culture flasks by previously described methods (34). Serial dilutions of fluids containing MCMV were made in maintenance medium containing medium 199 (GIBCO Laboratories, Grand Island, N.Y.), 3% fetal calf serum, 200 U of penicillin per ml, 200 μg of streptomycin per ml, and 0.525% sodium bicarbonate. Appropriate dilutions were added in 0.5-ml volumes to each of two cell monolayers, and virus was allowed to adsorb for 2 h at 37°C. The inoculum was removed, and monolayers were overlayed with 0.8% gum tragacanth (Sigma Chemical Co., St. Louis, Mo.) in maintenance medium, incubated for 5 days at 37°C, washed successively with 0.85% saline, fixative containing ethanol, acetic acid, and 10% formalin (6:2:1), and 1% crystal violet in water. Plaques were counted with the aid of a dissecting microscope.

Mice. Outbred CD-1 mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were 5 to 8 weeks old at the time of use. Inbred C_3H/HeJ mice were obtained from Jackson Laboratories, Bar Harbor, Me., and were used when they were 6 to 10 weeks old. Mice were given various regimens of virus or CY (Cytoxan; Mead Johnson & Co., Evansville, Ind.) or both intraperitoneally, and mortalities were recorded daily for 20 days after infection.

Detection of virus in target organs. In some cases, lung, liver, and spleen were removed from infected mice, and 10% (wt/vol) homogenates were made with sterile sand, mortar, pestle, and maintenance medium. Extracts were clarified by low-speed centrifugation, 10% dimethyl sulfoxide was added, and small portions were stored at −70°C. Organ extracts were assayed for virus in the same manner described for virus pools.

Histological preparations. Lungs were fixed in situ, as previously described (32). Spleen, liver, and thymus were removed from the animal, cut in small pieces, and placed in fixing solution (0.103 M glutaraldehyde, 0.1125 M sodium cacodylate). The solution was changed once, and tissues were stored in the same fixative. Tissues were processed by the standard paraffin technique for histopathological evaluation.

Lymphocyte response to mitogens. Spleen cells from uninfected mice treated with 150 mg of CY per kg of body weight and untreated mice were removed, and responsiveness to phytohemagglutinin (PHA) (Wellcome Research Laboratories, Beckenham, England) and bacterial lipopolysaccharide (LPS) (*Escherichia coli* O128:B12; Difco Laboratories, Detroit, Mich.) was assessed in vitro by uptake of [^{3}H]thymidine. This assay was performed as previously described (31) except that erythrocytes were removed from spleen cell suspensions by using lysing buffer (0.15 M NH_4Cl, 0.1 M $KHCO_3$). For a given spleen, the mean of counts from triplicate microtiter wells containing medium and cells was subtracted from the mean of counts from triplicate wells containing mitogen and cells to obtain counts due to mitogen stimulation.

Assay for NK cell activity. YAC-1 cells were labeled by suspending 5×10^6 cells in 0.1 ml of RPMI 1640 supplemented with 10% fetal calf serum and adding 2 mCi of ^{51}Cr as sodium chromate in 0.1 ml of saline (New England Nuclear Corp., Boston, Mass.). The suspension was incubated for 1 h at 37°C in 5% CO_2 with gentle agitation at 10-min intervals. Cells were then pelleted by low-speed centrifugation, washed three times with RPMI 1640, and resuspended in RPMI 1640 plus 10% fetal calf serum (10^5 cells per ml). Spleen cells were prepared by the same methods used for the mitogen assay, suspended in RPMI 1640 (5×10^6 cells per ml), and dispensed in triplicate V-bottom microtiter wells (0.1 ml/well). To measure spontaneous release, 5×10^5 unlabeled YAC-1 cells in 0.1 ml were substituted for spleen cells; to measure total release, 0.1 ml of 0.5% Triton X-100 (Sigma) in distilled water was substituted for the spleen cell suspension. The microtiter plate was centrifuged at $300 \times g$ for 5 min, incubated for 4 h at 37°C in 5% CO_2, and centrifuged again. The amount of ^{51}Cr in 0.1-ml samples of supernatant from each well was determined with a Beckman Gamma 4000 counting system. The specific cytolysis was calculated by averaging the counts for triplicate wells and applying the following formula:

$$\% \text{ cytolysis} = \frac{\text{mean counts with spleen cells} - \text{mean spontaneous release}}{\text{mean total release} - \text{mean spontaneous release}} \times 100$$

RESULTS

Effect of CY on MCMV-induced mortality. To determine when infected mice would be most vulnerable to immunosuppression, groups of 14 to 16 CD-1 mice were treated with a single intraperitoneal injection of either 150 or 100 mg of CY per kg, given on various days before or after infection with 10^5 PFU of MCMV. Figure 1 shows that little or no mortality was observed in animals treated with CY 1 or 2 days before infection or 6 to 9 days after infection. Maximum mortality (80 to 90%) was observed when CY was given 2 to 4 days after infection. Most animals died 8 to 14 days after infection; those which received CY 4 days after MCMV tended to die later than those which received CY 2 days after MCMV. No deaths occurred among mice which were infected with 10^5 PFU of MCMV alone or among uninfected mice treated with 150 mg of CY per kg.

Figure 2 shows the effect of various doses of CY given 3 days after MCMV infection. Maximum mortality was observed when CD-1 mice were given 100 or 150 mg of CY per kg. There was no difference in the percent mortality seen in these two groups. The lowest effective concentration tested was 80 mg of CY per kg.

Figure 3 shows the effect of varying the virus dose on mortality observed in groups of mice given 150 mg of CY per kg 1 or 2 days before infection or 3 days after infection. As previously indicated, mortality was enhanced when CY was given 3 days after infection of CD-1 mice with 10^5 PFU but not when it was given 1 day before infection. Mortality was also enhanced when CY was given 3 days after infection with 4×10^4 PFU but not when lower doses of virus were given. The effect of giving CY before infection with these lower doses was not tested, since no effect was seen when CY was given before infection with 10^5 PFU. By raising the virus dose to 2×10^5 PFU, enhanced mortality could be seen in mice given CY 1 day before infection as well as in those given CY 3 days after infection. The latter group showed the greatest enhancement (100% mortality) over non-CY-treated controls (4% mortality). Elevating the virus dose

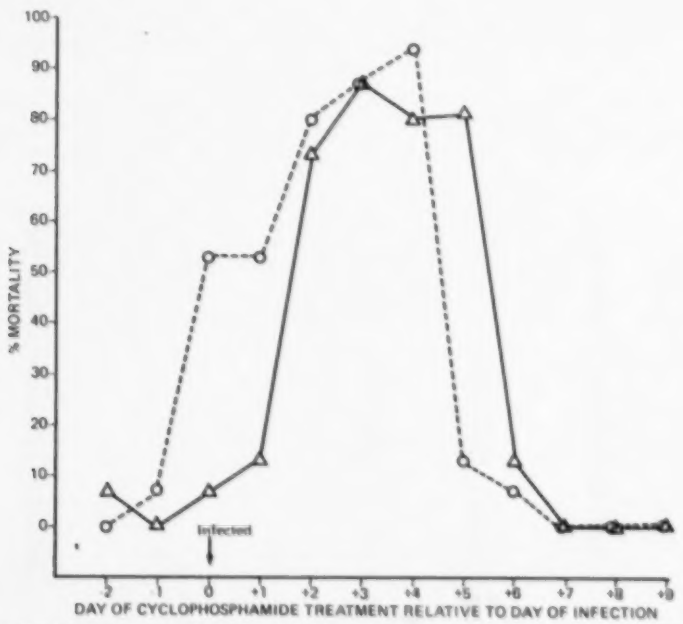

FIG. 1. Mortality in mice given a single dose of either 150 (○) or 100 (△) mg of CY per kg of body weight on various days relative to the day of infection with 10^5 PFU of MCMV. Each data point represents the percent mortality among a group of 14 to 16 mice given CY on the indicated day before or after infection.

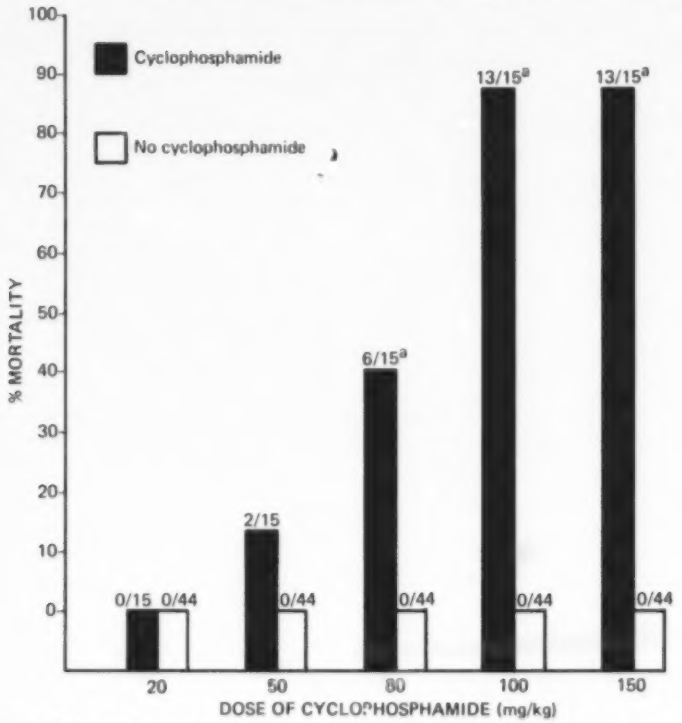

FIG. 2. Effects of dose of CY on enhancement of MCMV-induced mortalities. Groups of 15 mice were treated with doses ranging from 20 to 150 mg/kg 3 days after infection with 10^5 PFU of MCMV. a, $P < 0.01$ with a nonparametric Williams test. Numbers over bars represent number of mice dead out of number treated.

still further to 10^6 PFU increased the mortality seen in animals treated with CY 1 day before infection. However, because the number of deaths in untreated animals also increased, the difference in mortality between groups treated with CY 1 day before infection and untreated

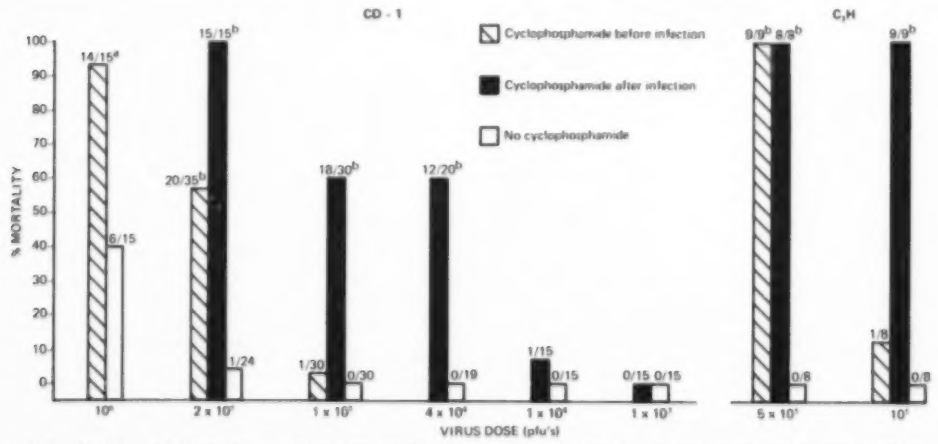

FIG. 3. Effect of virus dose on CY enhancement of mortality. CD-1 or C_3H mice were given 150 mg of CY per kg at various times relative to infection with various doses of virus. a, $P = 0.003$; b, $P = 0.0001$ when compared to open bars with Fisher's exact test. Numbers over bars indicate number of mice dead out of number treated.

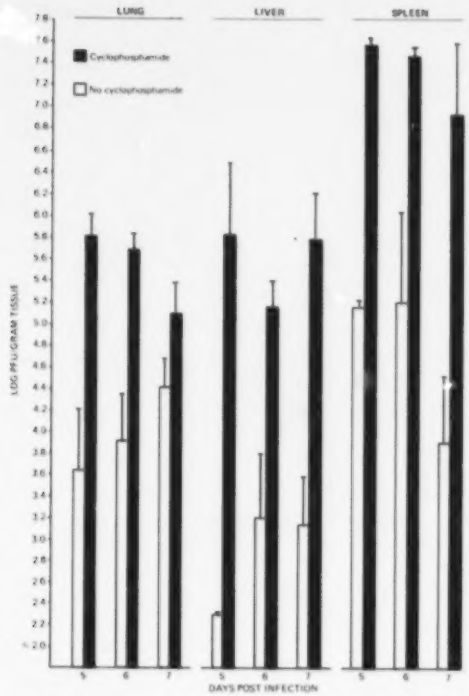

FIG. 4. Virus titers in target organs of mice infected with 2×10^5 PFU and given 150 mg of CY per kg 2 days after infection or left untreated. Each data point represents the mean and standard error of the log of PFU per gram tissue for three to six mice.

groups was not statistically different when the virus dose was 10^6 PFU as compared with 2×10^5 PFU.

Similar results were seen when C_3H mice were infected with 5×10^5 or 10^5 PFU and treated with 150 mg of CY per kg either 2 days before or 3 days after infection (Fig. 3). Compared with untreated controls, animals given the lower dose of virus showed enhanced mortality only when CY was given after infection. At the higher dose, both treatment regimens resulted in greatly enhanced mortality compared with untreated controls.

Effects of CY and MCMV infection on target organs. The effect of CY on production of virus in various target organs was determined by treating CD-1 mice with 150 mg of CY per kg 2 days after infection. Infected mice treated with CY or untreated were sacrificed, lungs, livers, and spleens were removed, and virus titers were determined for individual organs. The effects of CY treatment on virus titers in organs removed 5, 6, and 7 days after infection were analyzed by using analysis of variance (Fig. 4). In all organs, CY treatment caused an increase ($P < 0.0005$) in virus titers. This effect was most dramatic in the liver and spleen, in which for all days tested there was a 100- to 1,000-fold increase in the mean PFU per gram of tissue in CY-treated animals compared with untreated animals. This magnitude of increase was also seen in lungs taken 5 days after infection, but differences in lungs diminished to 5 to 50-fold on subsequent days. A significant interaction between CY treatment and day of titration postinfection was not seen, with the possible exception of the lung in which this interaction was marginally significant ($P = 0.09$) due to the lack of an effect of CY 7 days postinfection.

To determine whether the enhanced mortality seen in infected animals treated with CY was reflected in organ weights and histopathology, CD-1 mice infected with 10^5 PFU and treated with 150 mg of CY per kg 3 days after infection were sacrificed 5, 7, and 10 days after infection along with mice which received virus only, mice which received CY only, and untreated controls. Lung, liver, spleen, and thymus were removed from all animals, the latter three organs were weighed, and samples of organs removed on days 5 and 7 after infection were prepared for histopathological study.

After MCMV infection, spleen and liver weights were slightly higher than those of uninfected normal animals (Fig. 5), but differences between these two groups were never statistically significant. At all time points, whole body weights, as well as the weights of spleen and thymus, were significantly depressed in animals treated with MCMV plus CY compared with both untreated infected and normal uninfected mice. Liver weights in mice treated with MCMV plus CY were significantly depressed on days 7 and 10 postinfection. Some of this difference could be attributed to CY treatment alone, since body and organ weights were significantly depressed in this group 5 days after infection, but body, liver, and thymus weights in animals treated only with CY were not significantly different from controls on days 7 and 10 after infection, and spleen weight was not significantly different on day 10.

There were no notable microscopic lesions in any of the lung sections from any of the treatment groups. No significant lesions were noted in the livers of untreated mice or mice given CY only. Scattered pyknotic, degenerate hepatocytes (some of which contained intranuclear inclusions), focal collections of mononuclear cells, and Kupffer cell hyperplasia were observed in mice given MCMV or MCMV plus CY. Differences between these two groups were not striking despite the difference noted in liver

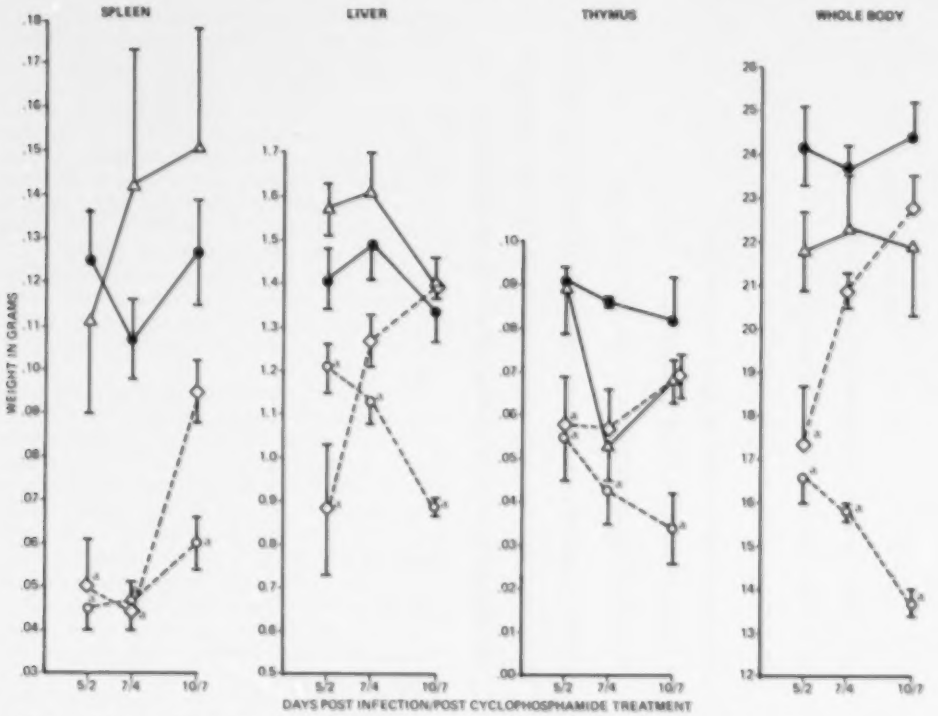

FIG. 5. Effects of CY treatment and MCMV infection on wet weight of organs. Each data point represents the mean and standard error of organ or body weights from seven mice. Symbols: △, 10^5 PFU of MCMV; ○, 10^5 PFU of MCMV plus 150 mg of CY per kg 3 days after infection; ◇, 150 mg of CY per kg; ●, no treatment. a, Significantly different from control ($P < 0.05$ with Dunnett's test) by using the error estimate from a two-way analysis of variance in randomized blocks.

weights. In the spleen, CY or MCMV produced a minimal to moderate lymphocyte depletion. Lesions caused by MCMV were slightly more severe and included a reticular-like cell hyperplasia and scattered necrotic cells throughout the parenchyma. When MCMV and CY were given to the same mouse, lymphocyte depletion was more severe, and red pulp necrosis, lymphocyte necrosis, and intranuclear inclusions in reticular cells were also observed. This picture is consistent with the observed drop in spleen weights in this group. Because of its central role in the immune response, the thymus was examined in these studies, even though it is not notable as a target organ in the sense of MCMV production (M. Selgrade, Ph.D. thesis, University of Wisconsin, Madison, 1973). MCMV caused minimal lymphocytic depletion, whereas CY and CY plus MCMV caused moderate to severe lymphocytic depletion. This is also consistent with the differences observed in thymus weight.

Effects of CY on immune responses. Uninfected mice were treated with 150 mg of CY per kg, spleens were removed on various days after infection from CY-treated and control mice, and the responses of lymphocytes from these two groups to PHA and LPS were determined and compared (Table 1). The effect of CY on responses to both mitogens appeared to be biphasic. One day after CY treatment, responses were depressed. With each successive day up to 4 days, the degree of depression diminished such that there was no difference between treated and untreated mice on day 4. However, 9 days after treatment, significant depression in both mitogen responses was again observed. Again, the degree of depression diminished on successive days; recovery from this second phase of depression was more rapid for LPS than for PHA.

The effects of CY treatment on MCMV-induced nonspecific cytotoxic activity of spleen cells were also assessed by using the ^{51}Cr release assay typically employed to detect NK cell activity. C_3H mice were treated with 150 mg of CY per kg 2 days before or 1 or 3 days after

TABLE 1. Effects of CY on mitogen response of spleen cells from uninfected mice

Days after CY treatment	PHA			LPS		
	Control (cpm × 10^4)[a]	CY-treated (cpm × 10^4)[a]	% Depression due to CY	Control (cpm × 10^4)[a]	CY-treated (cpm × 10^4)[a]	% Depression due to CY
1	5.50 ± 0.74 (6)	1.36 ± 0.40 (9)[b]	75	7.22 ± 1.17 (6)	0.57 ± 0.23 (9)[b]	92
2	7.74 ± 1.22 (4)	3.88 ± 0.71 (7)[c]	50	7.92 ± 0.91 (12)	3.53 ± 0.56 (12)[b]	55
3	8.08 ± 1.56 (7)	4.77 ± 1.29 (7)[c]	41	8.13 ± 1.23 (7)	5.01 ± 1.37 (7)[b]	38
4	7.98 ± 2.05 (5)	6.68 ± 0.65 (6)	16	9.28 ± 0.98 (6)	7.41 ± 0.81 (6)	20
9	11.57 ± 1.08 (7)	1.20 ± 0.31 (8)[b]	90	10.50 ± 1.22 (7)	5.09 ± 1.14 (8)[b]	52
12	4.72 ± 0.98 (6)	1.08 ± 0.41 (7)[b]	77	4.20 ± 0.31 (11)	3.46 ± 0.51 (12)	18
15	8.18 ± 1.20 (10)	6.48 ± 0.95 (12)	21	8.63 ± 0.60 (11)	6.59 ± 0.83 (12)	24
19	8.47 ± 1.09 (11)	7.02 ± 1.34 (11)	17	7.38 ± 1.63 (11)	6.99 ± 1.72 (11)	5
23	14.03 ± 1.39 (9)	10.69 ± 1.40 (9)[c]	24	11.31 ± 0.87 (9)	11.92 ± 1.08 (9)	0

[a] Mean ± standard error of net cpm (cpm for mitogen stimulated cells − cpm for unstimulated cells) for number of animals indicated in parentheses.

[b] Depressed when compared with control ($P < 0.05$).

[c] Depressed when compared with control ($P < 0.10$). Statistical methods were the same as those used for Fig. 5.

infection with 10^4 PFU of MCMV. Figure 6 compares the cytolytic activity in spleen cells from untreated mice and CY-treated mice on various days after infection. This activity in animals treated with CY 2 days before infection was significantly depressed on days 1 and 3 after infection but had recovered and reached peak activity at the same time untreated mice reached peak activity 5 days after infection. In contrast, NK activity in animals treated with CY 1 and 3 days after infection never reached the peak seen on day 5 in untreated animals. Activity in these animals was profoundly depressed on day 5 and remained depressed on day 8, although the difference between untreated and treated was not as great due to the normal waning of NK activity at this time. Similar trends were observed when animals received 10^5 PFU of MCMV, which caused a higher level of NK cell activity in untreated animals than was observed after infection with 10^4 PFU.

DISCUSSION

The data presented here show the effects of several different regimens of CY treatment on MCMV infection and MCMV-related parameters. Enhanced mortality was demonstrated in animals given single doses of CY ranging from 80 to 150 mg per kg. Such enhancement was more efficiently achieved when animals received CY after virus challenge. Our data on mortality are similar to those of Peller and Goetz (24), who demonstrated enhanced mortality and increased virus titers in the liver in MCMV-infected mice given a single dose of 80 mg of CY per kg on the day of infection or three doses given on days −2, −1, and 0 relative to infection. Agatsuma (1) also demonstrated enhanced mortality in MCMV-infected mice when three doses of 200 mg of CY per kg were given on days 7, 11, and 15 after infection.

In addition to enhanced mortality, virus titers in lung, liver, and spleen were elevated by CY treatment, supporting the thesis that deaths occur as a result of CY enhancement of MCMV infection in these vital organs rather than MCMV enhancement of CY toxicity. Also, the wet weights of liver, spleen, and thymus in infected mice treated with CY were depressed compared with untreated infected mice and, at some time points, with uninfected CY-treated mice. Although gross differences in histopathology were minimal, decreased liver and spleen weights in CY-treated, infected animals were probably due to more extensive virus-induced necrosis and a depressed inflammatory response. The depression of spleen and thymus weight in CY-treated animals with and without virus probably also reflects loss of leukocytes due to CY depression of bone marrow function. The fact that spleen and thymus weight in animals given MCMV plus CY remained depressed longer than in animals given CY alone suggests that MCMV somehow prolongs recovery of these organs to normal weight after CY treatment and therefore may act synergistically with CY in depressing thymic and splenic functions. Decreased body weight in these animals probably reflects a general cachexia due to the severity of the infection.

The effects of CY on production of MCMV-neutralizing antibody were not examined, since such antibody is normally not detected until 14 to 33 days after infection (23) and therefore could not have affected the outcome in this study, in which mice died 10 to 14 days after infection. Because mitogen-induced lymphocyte proliferation is often used to test compounds for immunotoxicity, the effects of CY on the response of lymphocytes to T (PHA) and B (LPS) cell mitogens were included in this study to determine whether such tests are predictive of

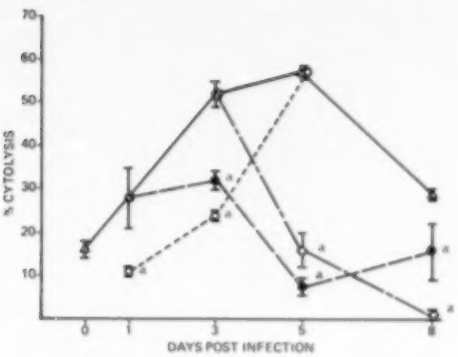

FIG. 6. Effect of 150 mg of CY per kg given on various days relative to infection on MCMV-induced NK cell activity. Symbols: △, virus only; ○, CY given 2 days before infection; ●, CY given 1 day after infection; ◇, CY given 3 days after infection. Data points represent the mean ± standard error for three to six mice. a, Significantly different from virus-only group ($P < 0.05$). On day 8, the activity in the group treated with CY on day 3 was significantly lower than that of mice treated with CY on day 1. Statistical analysis was performed by using a one-way analysis of variance for each day tested. For days 3, 5, and 8, treatment effects were subtested with Duncan's multiple range test.

enhanced susceptibility to MCMV. Uninfected mice were used because MCMV itself greatly depresses responses to PHA and LPS for 2 to 3 weeks after infection (31). Both mitogen responses were initially depressed by CY treatment, recovered with time after treatment, and then showed a second phase of depression and recovery. This biphasic response to CY may reflect the fact that more than one cell type is involved in the mitogen response. These may turn over at different rates and thus be affected at different times after CY treatment.

Figure 1 predicts that CY depression of the immune responses relevant to MCMV susceptibility must be fairly short-lived, since mice given CY 1 day before infection were unaffected. If the depression of mitogen responses on various days after CY treatment had not been biphasic (Table 1), a good correlation between depressed mitogen responses and enhanced susceptibility would have been obtained. However, the second phase of depressed mitogen responses, which began around day 9, mars this correlation. Depressed mitogen responses were observed at many of the time points up to 9 to 12 days after CY treatment, a period of time adequate to cover the entire acute stage of MCMV infection, even in animals given CY 1 day before infection. The importance of the time of administration of CY relative to infection as it affects the outcome of the disease (Fig. 1) could be more easily explained by the effect of the time of CY administration on MCMV-augmented nonspecific cytolytic activity (Fig. 6). Animals treated with CY 2 days before infection recovered this activity apparently in time to recover from the disease, whereas those treated with CY 1 and 3 days after infection did not recover this cytolytic activity and experienced a more severe disease.

Without further attempts to characterize the cell type responsible for target cell lysis, we assume that the activity we observed was due to NK cells for several reasons. The cytotoxicity was directed against non-H-2-related cells in the absence of viral antigen, eliminating cytotoxic T cells as a possible effector. Also, the curve obtained for untreated mice in Fig. 6 corresponds very well to that obtained for NK cell activity by Quinnan and Manischewitz (26). In contrast, it does not correspond to a curve obtained by Schleupner et al. (30), in which enhanced macrophage cytotoxicity for tumor cells after MCMV infection reached a peak 3 days postinfection and remained at this level until approximately 21 days postinfection.

Quinnan and Manischewitz (26, 28) were the first investigators to suggest an important role for NK cells in host defenses during the acute stage of MCMV infection. Bancroft et al. (3) provided more evidence in support of this thesis by demonstrating a significant correlation between resistance to MCMV and the degree of NK cell augmentation by the virus in 10 strains of susceptible and resistant mice. The temporal relationship demonstrated in the present study between time of CY administration, enhanced mortality, and decreased NK cell activity provides more evidence that NK cells have an important influence on the outcome of acute MCMV infection. The relationship observed here between time of CY administration and depression of NK cell activity correlates well with data presented by Riccardi et al. (29) which showed maximum depression of spontaneous NK cell activity 4 days after treatment with 240 mg of CY per kg.

It has been demonstrated that immunosuppressive treatment with radioactive strontium enhances MCMV infection without depressing NK cell activity (17). In addition to NK cell activity, several other immune defense mechanisms are thought to influence the outcome of MCMV infections. These include macrophage activity (35), cytotoxic T cell activity (27), and interferon production (16). Any treatment which reduced these responses might also enhance mortalities and virus target organ titers due to MCMV. This illustrates the need to include tests for immunological function along with disease susceptibility tests in any tier of tests designed

to assess immunotoxicity. These are needed to determine what aspects of the immune response are affected by a particular treatment and to verify that enhanced mortality is a result of immunosuppression. It also suggests that although NK cell activity may play an important role in the host defense against MCMV, there are other immune responses which also contribute to host protection.

From our data, we conclude that the effects of CY on MCMV susceptibility corresponded well with effects on thymus, spleen, and liver wet weight and virus titers in lung, spleen, and liver and with CY effects on virus-induced NK cell activity. Good correlation was not obtained between effects of CY on MCMV susceptibility and CY effects on responses of lymphocytes to B and T cell mitogens, nor were differences in pathology particularly striking. NK cells are thought to play an important role in host defenses against tumor cells as well as other viruses. In addition, other immune responses thought to be important host defenses against MCMV, such as macrophage activity, cytotoxic T cell activity, and interferon production (16, 27, 35), are also thought to have a role as antitumor defense mechanisms (11). Susceptibility to MCMV, coupled with an assay for NK cell activity, as well as some of the above-mentioned immune responses, might therefore be very useful parameters to include in a series of tests designed to screen chemicals for immunotoxicity. MCMV would provide a disease susceptibility test which could be assessed much more rapidly than increased tumor susceptibility and could perhaps provide similar answers.

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